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Illuminating Autoimmune Regulators through Controlled Variation of the Mouse Genome Sequence

Review

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Gene variants in mice that have strong, Mendelian effects on autoimmune susceptibility have been one of the most productive entry points for identifying genes and processes regulating human autoimmunity. With the tools now available to map and identify new mouse Mendelian gene variants, the handful of spontaneous mutations accumulated over several decades have all been identified, and the main bottleneck lies in producing new Mendelian immune variants. We outline here a strategy to generate large sets of functional variants in genes controlling lupus and humoral immunity, based upon limited variation of the mouse genome sequence with the chemical mutagen, ENU, combined with a set of sensitive immunological screens.

Toward a Rationally Designed Therapy for Systemic Autoimmunity

Global immunosuppression is still the mainstay of therapy for most systemic autoimmune diseases. This is almost always associated with toxicity which can be life threatening and often with lack of efficacy because the treatments lack specificity. The search for more specific therapeutic agents will depend on obtaining a precise understanding of pathogenesis (Goodnow, 2001). One approach to this goal is to identify genetic variants that are consistently associated with disease phenotypes and define the function of those genes. Already, biological agents that target molecules directly involved in disease pathogenesis provide remarkable therapeutic efficacy with much less toxicity. A clear example has been the remarkable effectiveness of TNF-neutralizing agents in rheumatoid arthritis (RA), which is at least as efficacious as standard disease-modifying regimens and is highly effective at controlling symptoms of arthritis. TNF antagonists entered clinical trials after identification of TNF as a key mediator of synovial inflammation and local bone demineralization in preclinical mouse models and clinical samples, reports of polymorphisms in the TNF gene associated with RA, and studies showing prompt amelioration of symptoms in mouse models of arthritis following administration of monoclonal antibodies to TNF (Feldmann and Maini, 2001). This success illustrates the importance of identifying new autoimmune alleles in order to design more effective, less toxic therapies.

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Identifying Autoimmune Disease Genes: A Signal to Noise Ratio Problem

Over the past 15 years, considerable efforts worldwide have been devoted to mapping the genetic loci associated with autoimmunity (Kelly et al., 2002; Mohan, 2001; Todd and Wicker, 2001). In human population studies or crosses between different strains of mice, over 50 lupus-susceptibility linkage regions have been found, but it has proved very difficult to identify the causative gene variants themselves. Two factors contribute to this difficulty. First, there appear to be many different loci contributing additively, epistatically, or heterogeneously to the autoimmune phenotype, so that individual genotype-phenotype correlations represent a weak signal to trace. Second, there is a high background of noise in the form of natural sequence variation—roughly one base change in every thousand between different humans or rodent strains—so that every gene in a mapped interval typically contains multiple sequence variants. In humans with organ-specific autoimmunity, the combination of massive population studies, linkage disequilibrium analyses, and biological analysis of plausible candidate genes has been able to overcome these problems in several notable cases (Bennett et al., 1995; Morahan et al., 2001; Pugliese et al., 1997; Ueda et al., 2003; Vafiadis et al., 1997). In mice, a productive solution to this signal to noise problem has been to phenotype a set of genotypically identical replicates through breeding of congenic substrains (Morel and Wakeland, 2000; Rozzo et al., 2001; Silveira et al., 2001; Todd and Wicker, 2001). Global mRNA profiling of congenic mouse tissues on microarrays has proved a valuable additional filter to tease out candidate genes within a congenic interval (Eaves et al., 2002; Rozzo et al., 2001), although it will only succeed for gene variants with altered mRNA abundance.

Autoimmune regulatory genes and mechanisms that have so far been identified have almost all come by analyzing sequence variants with a stronger genotype-phenotype relationship—primarily spontaneous Mendelian mutations or gene-targeted knockouts (Plotz, 2003; Theofilopoulos and Kono, 1999). For example, spontaneous mouse mutations in Fas (Watanabe-Fukunaga et al., 1992) and FoxP3 (Brunkow et al., 2001) have been key entry points for delineating new immunoregulatory processes and guided identification of corresponding human mutations and definition of specific autoimmune syndromes such as ALPS (autoimmune lymphoproliferative syndrome) and IPEX (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked; Bennett et al., 2001; Chatila et al., 2000). Similarly, human mutations in complement C4 or C1q (Meyer et al., 1985), AIRE (autoimmune regulator; Nagamine et al., 1997; Finnish-German APECED Consortium, 1997), and NOD2 (Hugot et al., 2001; Ogura et al., 2001) have, respectively, revealed unexpected pathways for clearance of apoptotic cells (Botto and Walport, 2002), thymic deletion to organ-specific antigens (Anderson et al., 2002; Liston et al., 2003), and regulating immune/inflammatory responses to cell wall components of gut bacteria (Inohara

and Nunez, 2003). Spontaneous mutations in SHP-1 (Shultz et al., 1993; Tsui et al., 1993), followed by targeted mutations in lyn, CD22, FcgR2, CD5, CTLA4, and PDCD1, have delineated the importance of tuning antigen receptor thresholds via inhibitory feedback signaling through ITAM-containing receptors and tyrosine phosphatases (reviewed in Goodnow, 2001; Chambers et al., 2001; Greenwald et al., 2002; Okazaki et al., 2002a; Ravetch and Lanier, 2000). Definition of these genes as autoimmune regulators has made them productive candidates for identifying human autoimmune susceptibility variants (Prokunina et al., 2002; Ueda et al., 2003). Similarly, targeted knockouts have established the autoimmune regulatory and receptor tuning role of the ubiquitin ligase, cbl-b (Bachmaier et al., 2000; Chiang et al., 2000), enabling candidate-based identification of a diabetes-causing mutation in rats (Yokoi et al., 2002).

The Autoimmune Mouse Variant Bottleneck

While spontaneous or targeted Mendelian autoimmune mouse variants have been the most productive route to identifying and understanding autoimmune regulators, production of new variants now represents the chief bottleneck to further progress. The human and mouse genomes are currently 95% sequenced, and the coding sequence for greater than 80% of genes is annotated, setting out orthologous human and mouse genes (Okazaki et al., 2002b; Waterston et al., 2002). This remarkable set of sequence blueprints, coupled with the powerful tools for fine-mapping Mendelian mutations in mice, removes many previous barriers to identifying autoimmune regulators. More than three quarters of mammalian genes have nevertheless not yet been attributed a biological function (Nadeau et al., 2001). Moreover, even for genes that already have well-established roles in lymphocyte activation, such as ZAP-70, LAT, or Carma-1, specific-sequence variants at key residues can alter their function in ways that cause hypersensitivity/autoimmunity rather than immunodeficiency (Aguado et al., 2002; Jun et al., 2003; Sakaguchi et al., 2003; Sommers et al., 2002). The handful of spontaneous autoimmune or immunodeficient mouse mutants accumulated during the pregenomic period has already been mapped and sequence variants identified. Postgenome, the chief gap now lies at the front end of genetic analysis, in producing new Mendelian mouse variants with autoimmunity or altered immune responsiveness.

Targeted production of new mouse variants, either by homologous recombination in ES cells or by gene knockdown with RNAi, provides only a partial solution to the mutant bottleneck. These gene-driven strategies tend to be biased toward a narrow band of candidate genes of known immunological relevance, and limited understanding of complex processes such as self-tolerance or immune regulation makes identification of new candidate genes difficult. A further difficulty lies in the fact that autoimmune-prone knockout mice are usually generated on a 129/Sv genetic background, a strain with a confounding intrinsic propensity to develop autoimmunity and autoantibodies (Santiago-Raber et al., 2001). Gene knockout or knockdown strategies commonly aim to eliminate whole proteins, as opposed to natural sequence variation which most often yields missense alleles that selectively disturb specific protein domains. As illustrated by point mutant alleles in Zap70, LAT, Carma-1, and Ikaros (Aguado et al., 2002; Jun et al., 2003; Papathanasiou et al., 2003; Sakaguchi et al., 2003; Sommers et al., 2002), elimination of an entire protein can obscure key immunoregulatory functions either because development of the mouse, cell lineage, or cell response is abolished altogether or because another related protein fills the vacant niche. Here, we review the background and progress addressing this bottleneck via a complementary strategy: by introducing a limited number of single base variants into a homogenous non-autoimmune mouse strain, coupled with screens to reveal variants in autoimmune regulators.

Controlled Production of Germline Sequence Variants with ENU

Repeated in vivo exposure of spermatogonial stem cells to the alkylating agent N-ethyl N-nitrosourea (ENU) introduces single base pair substitutions throughout the mouse genome at a rate estimated between one base in every 100,000 and one in two million (Beier, 2000; Coghill et al., 2002). More than 80% of ENU-generated germline mutations are either AT to TA transversions or AT to GC transitions (Popp et al., 1983). Of the variants that have a phenotypic effect, two-thirds interfere with gene function by changing a single codon to encode a different amino acid, and the rest cause either splicing errors or nonsense (stop) codons (Justice et al., 1999). These point mutations thus hold the unique attraction of altering discrete domains and functions of encoded proteins rather than creating simple null alleles. Based on the frequency of new loss-of-function alleles measured at a considerable range of loci, ENU treatment of male mice yields an average of one new loss-of-function mutation per gene in every 1000 gametes (Hitotsumachi et al. 1985; Beier, 2000; Coghill et al., 2002; Justice et al., 2000). Thus, if 500 to 1000 gametes were to be tested by a suitable breeding and immunological screening strategy, the majority of immunoregulatory genes that could be detected by the screen will have been represented by at least one loss-of-function variant. Once each variant has been recognized and successfully propagated to establish a true-breeding line, with chromosomally linked molecular markers to identify carriers, the process of distribution, analysis, fine-mapping, and sequencing each variant is relatively straightforward and represents a tremendous potential resource for the research community to link genome with "phenome." The front-end steps of recognizing immunological variants and establishing them as readily distributable strains nevertheless pose considerable challenges, which we address below.

Breeding Strategies to Identify Immune Variants

Most variants in immunoregulatory genes are loss-of-function alleles that have either recessive or semidominant effects on the immune system. To recognize them, sequence variants in the ENU-treated gametes need to be bred to homozygosity through a three-generation breeding program (Nelms and Goodnow, 2001) (see examples in Figures 2 and 4). The process starts with individual generation 1 (G1) offspring of ENU-

treated males. Each G1 mouse is estimated to carry an independent set of ~30 loss-of-function variants on the paternal chromosomes, based on the average frequency of new loss of function variants per gamete (1/1000) and the number of mammalian genes (~30,000). For immunological variants, two unrelated G1 mice are paired together, founding a pedigree with an estimated 60 functional variants including those on the paternally derived X and Y chromosomes. Their G2 offspring have a 50% chance of being heterozygous carriers for any variant present in their G1 parents, and five brother-sister pairs of these G2 animals are set up with the aim of achieving four productive pairs. One quarter of these G2 x G2 pairs will share any particular variant present in the pedigree, and one quarter of their G3 offspring (1/16 of all the G3s in a pedigree) will therefore be homozygous for this variant gene. By producing "libraries" of 100 such pedigrees each year—each pedigree founded with different G1 animals, yielding ~40 G3 offspring per pedigree and taking at least one year to complete—it is feasible to test an estimated 4000 loss-of-function gene variants for immune regulatory roles (68% of the ~6000 recessive variants present in the 200 G1 founders). The Australian government, in conjunction with the ANU John Curtin School for Medical Research, Monash University Institute for Reproduction and Development, The University of Queensland Institute of Molecular Bioscience, and the Garvan Institute, has established an open-access facility, the Australian Phenomics Facility, with infrastructure and database systems to support construction and testing of many such libraries in parallel (www.apf.edu.au).

The single biggest problem facing this strategy is the fact that identifying mice with variant alleles of autoimmune regulators is much less straightforward than picking out coat color variants. For this reason, only a handful of spontaneous immunological mutants have been collected in inbred mouse collections over several decades, in stark contrast to the large collections of visible traits. Large numbers of heritable new variants in autoimmune regulators can be recognized by serological and immunization tests, as described below, but these tests are inherently "noisy" with false positives due to nongenetic phenotypic variation. False positives can be reduced if one restricts the cutoff defining a putative mutant ("putant") to the most extreme variants, but this comes at an unacceptable cost of excluding most of the heritable variants which have clear but less extreme phenotypes.

The pedigree structure described above is designed to provide an alternative solution to this problem, by allowing familial clusters of heritable variants to be recognized in pedigrees. For example, if one sets a cut-off for interesting variants at the 95% tails of the normal distribution, false positives will occur in 5% of animals, and the chance of a second false positive in an immediate sibling is still 5%. By contrast, for a heritable recessive variant, 25% of siblings are also expected to be homozygous for the same variant, so that if ten G3 offspring are tested per G2 x G2 pair, it is likely that two homozygous variant siblings will be found. This familial clustering has proved to be a valuable filter for both the antinuclear antibody and immunization screens described below.

Immunization Screens

A battery of immunization tests has been developed and validated to identify variants in genes involved in antibody production. The aim of this screen has been to develop a resource of new strains that will identify new processes and immunosuppressive targets to inhibit autoimmune or allergic antibody responses or to enhance vaccination and immunity. Preimmune serum from each G3 mouse in each pedigree is first collected at ~8 weeks of age, and the mice are then immunized intraperitoneally with alum-precipitated chicken γ globulin coupled to the hapten arsonate (ABA-CGG) and heat killed *Bordetella pertussis* (BP) (Figure 1). The primary antibody response is measured by ELISA in serum 15 days later. The CGG response is dominated by IgG1 while the BP response is dominated by IgG2a (Toellner et al., 1998), providing an internally controlled test for the ability to polarize T cell-dependent antibody production to Th2 versus Th1. Figure 2 illustrates an example of a variant picked up by this screen and then successfully established as a true-breeding strain over the next 9 months by several generations of breeding and testing with the same immunization protocol. Four weeks after the first immunization, the mice are boosted with a second immunization of ABA-CGG and the T-independent type 2 antigen, NP-Ficoll, and the secondary immune serum is collected 7 days later and tested by ELISA. The titers of IgG antibody to ABA and CGG provide two separate measures of the capacity to undergo affinity maturation and humoral memory.

In a collaborative program between the ANU and Oxford University supported by the Wellcome Trust, a panel of 34 putative immune variants has been isolated in this way from 189 pedigrees in two sequential libraries, ENU4 and ENU5, derived from ENU-treated C57BL/6 mice (www.apf.edu.au/resources/wt/). Propagation and tracking inheritance of these variants, by immunization testing of offspring on the parental background and in mapping crosses with other inbred strains, is a key hurdle for establishing true-breeding lines and linked molecular markers to facilitate identification of carriers and homozygotes by other laboratories. Once each strain has reached this point, it is available for distribution to the research community and for expanded analysis of genes and immune processes. Development of a broad information base about immunological phenotypes and processes in each established strain will be achieved by free distribution to the research community on the condition that all new data obtained about each strain be deposited in the Resource database.

Screens for Systemic Autoimmune Disorders

Systemic and organ-specific autoimmune diseases are conducive to genetic dissection by mouse chemical mutagenesis, since the existing mouse autoimmune strains develop remarkably similar autoimmune manifestations to their human counterparts. Tests for new inherited variants in autoimmune regulators should be nonlethal, robust, and highly reproducible with less than 5% false positive rates, and affected animals should preferably be fertile. Unfortunately, in most complex autoimmune diseases, such as systemic lupus, overt disease tends to have incomplete penetrance as assessed by the

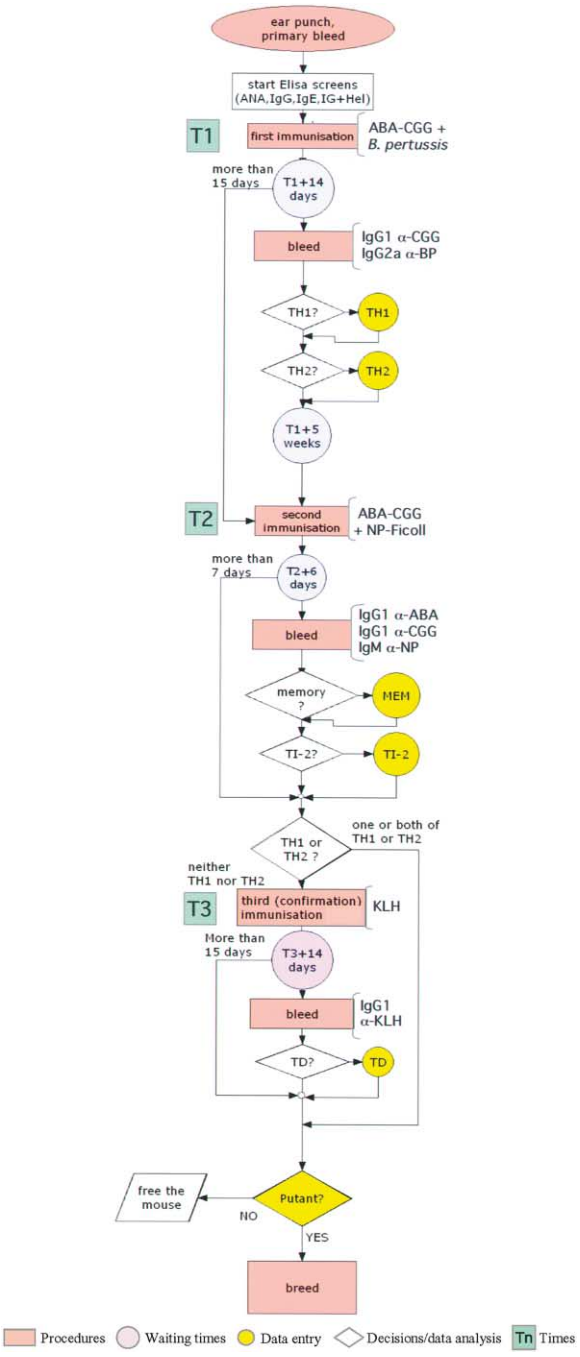

Figure 1. Humoral Immunity Screen

G3 ENU mice are bled at 12 weeks of age, and flow cytometric analysis of leukocyte subsets, ELISAs for total IgG and IgE, and immunofluorescence for ANAs are performed. Mice are then immunized with alum-precipitated ABA-CGG and heat-killed inactivated *B. pertussis*. Selective defects in IgG2a against *B. pertussis* or IgG1 against CGG identify Th1 and Th2 putants, respectively. Five weeks later mice are reimmunized with soluble ABA-CGG and NP-Ficoll. Selective defects in IgM against NP or IgG1 against ABA or CGG identify TI-2 or memory mutants, respectively. Mice that fail to produce an antibody-specific response to both CGG and *B. pertussis* are reimmunized with keyhole limpet hemocyanin (KLH) to exclude the possibility of failed primary immunization.

30%–40% lupus concordance in identical (monozygotic) twins (Kelly et al., 2002). This means that on many occasions the autoimmune phenotypes of new gene variants will be subtle or incompletely penetrant. A useful strategy is to identify and screen for variants in biomarkers that are well validated to precede development of overt disease and thus may be more penetrant than the full-blown clinical syndrome. This offers the additional advantage that it allows earlier identification of variant animals, saving time and increasing the likelihood that affected animals will breed, although it will also inevitably decrease specificity and raise the number of false positives. The problem of false positives can be overcome by the familial clustering strategy outlined above. Thus, finding clusters of several affected sibling animals increases the confidence in the heritability of the trait, before spending considerable effort in several generations of progeny testing.

Antinuclear antibodies (ANAs) are the most prevalent biomarker for systemic lupus and represent a sensitive but relatively nonspecific test for the disease. They are also found in other systemic autoimmune diseases (i.e., scleroderma and mixed connective tissue disorders), chronic bacterial and viral infections, chronic liver disease, and a number of malignancies. ANAs can also be found in 2% of healthy adults and are influenced by age and sex hormones, with a higher incidence in women and a further increase in those taking hormonal-based contraceptives (McMurray, 2003). A higher proportion of siblings of lupus patients compared to the normal population develop antinuclear antibodies, suggesting this serological trait can be more penetrant than development of clinical lupus. This conclusion is also borne out by analyses of congenic mouse substrains isolated from NZB/W mice (Mohan et al., 1999). ANAs develop first and may precede clinical manifestations by many years. Next, antibodies to SS-A and phospholipids occur. Finally, the more specific lupus-associated antibodies, such as anti-Smith (Sm) and dsDNA antibodies develop close to the onset of clinical symptoms (Arbuckle et al., 2003).

It follows that detection of antinuclear antibodies may be a sensitive surrogate assay for detecting an underlying genetic propensity to develop systemic autoimmunity later in life. Detection of ANAs in the serum is simple, inexpensive, non-invasive, and amenable for implementation in high-throughput screens of mice. When performed as an immunofluorescence-based assay it is a robust test for autoimmunity. However, due to their relatively low specificity for lupus, ANAs found during mouse ENU screens should be analyzed in the context of stringent criteria such as titer, age of onset (15–20 weeks), pattern of immunofluorescence, and clustering of at least two affected siblings. In the ANU/Oxford Wellcome Trust program, we have screened a library of 200 G3 pedigrees of mutagenized C57BL/6 gametes for IgG ANAs by immunofluorescence on Hep-2 cells. Both the intensity and pattern of staining were scored, complemented by urinalysis for proteinuria, glycosuria, hematuria, a FACS-based assay to detect T cell hyperactivation and regulatory T cell numbers, and serum analysis for hypergammaglobulinemia (Figure 3). This ANA-based screen yielded 29 putative autoimmune mouse variants (www.apf.edu.au/resources/wt/). Some exhibit an ex-

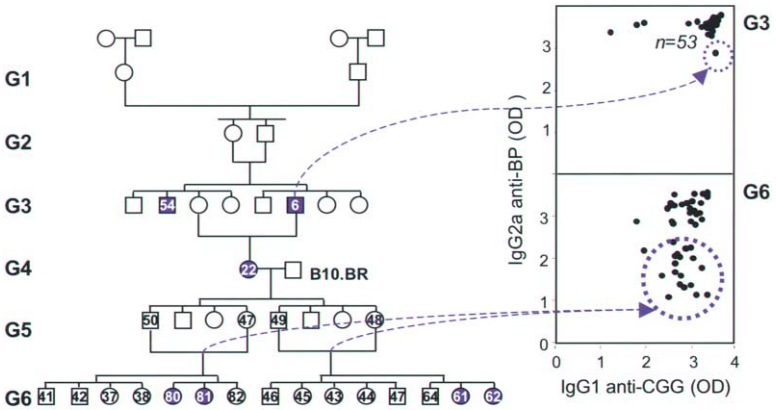

Figure 2. Putant Identification and Heritability Testing

Example of a variant (putant number 6) identified by the immunization screen as having a selectively decreased Th1 response (decreased IgG2a response to *B. pertussis* in the presence of normal IgG1 titers to CGG). The putant was bred to a carrier sibling and then established as a true-breeding strain by two further generations of breeding and testing with the same immunization protocol.

tended phenotype, such as lymphadenopathy, hematological cytopenia, proteinuria, and/or hypergammaglobulinemia. To date, heritability of eight strains has been confirmed, twelve are still in heritability analysis, five were unable to be propagated, and four were tested and found not to be heritable. Examples of ANA results and a family tree for a representative mutant are shown in Figure 4.

Validation of this strategy has come from the first strain to be identified by the ANA screen, *san roque*, which we have recently taken through fine mapping to a 1.5 Mb interval and sequencing each of the 15 transcription units within this interval. The mutation in *san roque* revealed a novel immunoregulatory gene, well-conserved across phyla but of previously unknown function (unpublished data). Based on detailed analysis of homozygous mutants, the gene identified is an essential negative regulator of T cell activity and cytokine produc-

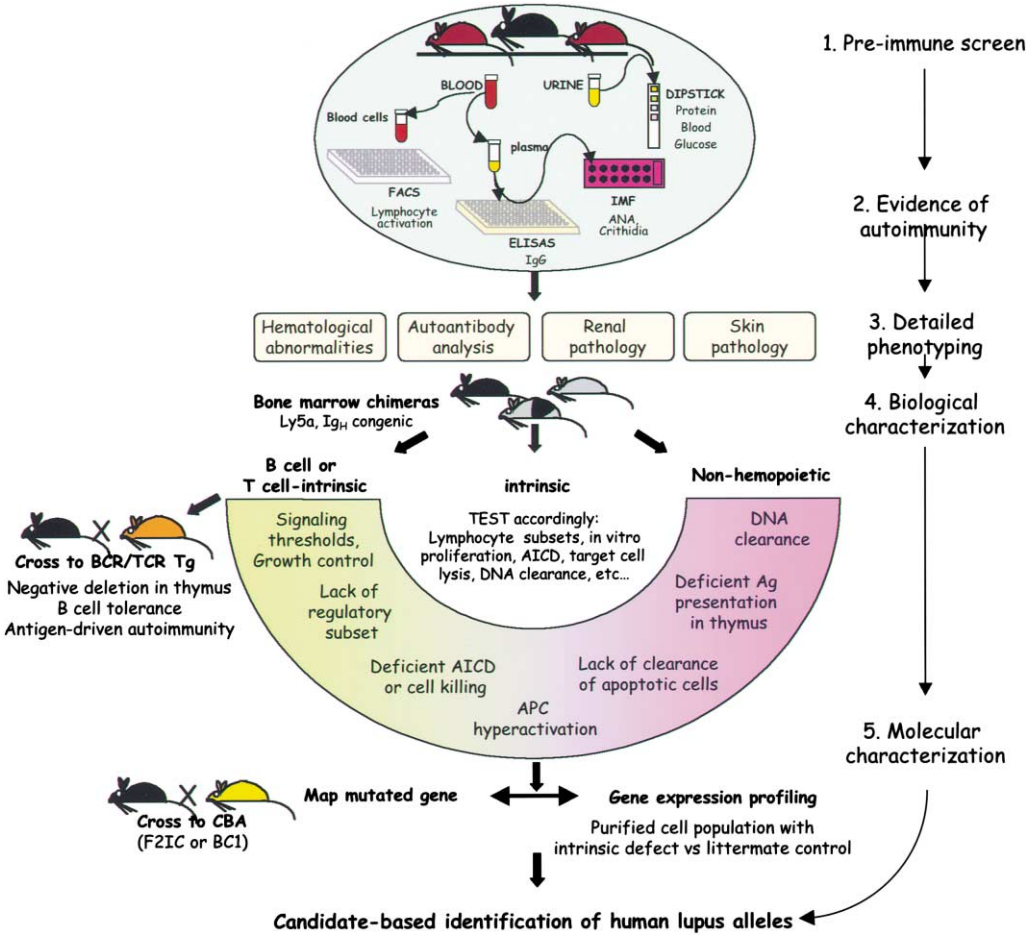

Figure 3. Systemic Autoimmunity Screen

Summary of the steps to identify and characterize ENU-induced systemic autoimmune phenotypes (see text).

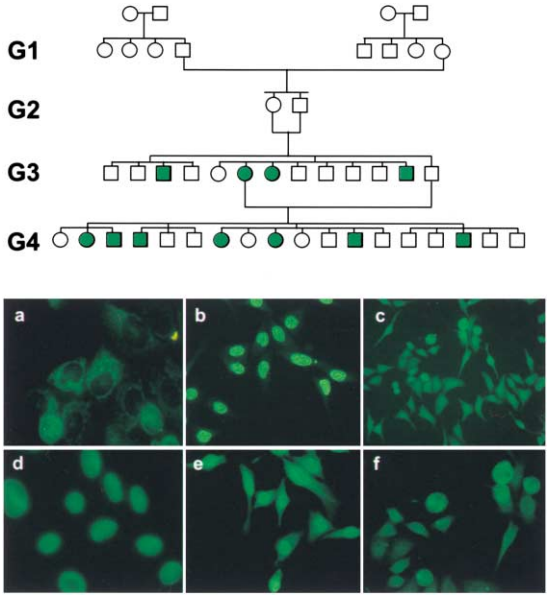

Figure 4. Heritability of ENU-Induced ANA Production and Patterns Identified

Top: Genealogy tree of a pedigree exhibiting a fully penetrant autosomal recessive ENU-induced mutation causing ANA production. Bottom: ANA patterns identified in ENU-mutagenized strains include: (A) possible mitochondrial, (B) homogeneous nuclear, (C) mixed nuclear and cytoplasm, (D) nuclear envelope, (E) cytoplasmic/vimentin, and (F) nuclear speckled.

tion. This strain provides proof of principle and a template for analyzing the variant genes in each of the new ANA autoimmune strains.

The "hit rate" for ANA-positive variants, one in every seventh pedigree, is surprisingly high compared with gross abnormalities in blood lymphocyte subsets which occur approximately once in every ten pedigrees. Nevertheless, the frequency is consistent with previous work revealing as many as 40 single gene knockouts that can cause ANAs and lupus-like diseases, involving defects in a broad range of cellular processes (Mohan, 2001). B cells specific for nuclear antigens are part of the normal repertoire and dysregulation of numerous pathways including T cell and B cell tolerization, activation thresholds, and killing or apoptosis of activated lymphocytes, and clearance of DNA or apoptotic cell debris have all been shown to lead to antinuclear antibody production (Mohan, 2001). Furthermore, there is accumulating evidence that properties that are shared and intrinsic to the autoantigens such as structure, glycosylation patterns, location after cell death, selective cleavage by granzyme B, and certain proinflammatory properties—i.e., intrinsic chemokine activity of various tRNA synthetases, complement fixation ability, or direct TLR9 activation by bacterial or self-DNA—can play direct roles in enhancing autoantibody production (Plotz, 2003). Multiple molecules in many different pathways are thus likely to play a role in regulating autoantibody production against systemic self-antigens, and the controlled production of new ANA variants provides a unique resource to investigate them.

Phenotypic and Functional Subsets of ANA Strains

Intriguingly, the different variant lines develop distinct nuclear and cytoplasmic autoantibody patterns that closely mimic those found in patients with systemic autoimmune disease (Figure 4). Some lupus manifestations and autoantibody profiles coincide with sufficient consistency in different subsets of patients to suggest that lupus is actually a cluster of related diseases. For example, Sm and C1q antibodies occur frequently with renal lupus, RNP antibodies with Raynaud's phenomenon and a low incidence of lupus nephritis, SSA antibodies with fetal heart block and extensive dermatitis, and phospholipid antibodies with thromboembolic disease and fetal loss. It can be inferred from this that detailed analysis of lupus serology is an important surrogate marker of critical pathophysiological events. These findings also suggest that different pathogenic pathways may contribute to different manifestations, implicating different genetic abnormalities. For example, C2 deficiency predicts a lupus phenotype characterized by marked photosensitivity, subacute cutaneous lupus, and the presence of SS-A antibodies (Meyer et al., 1985).

A positive ANA indicates the presence of autoantibodies, but in clinical practice further investigations and clinical findings are required for the diagnosis of systemic autoimmune diseases. Importantly, stratification of human lupus patients according to discrete clinical manifestations such as nephritis, thrombocytopenia, hemolytic anemia, neuropsychiatric disease, and associated rheumatoid arthritis or presence of anti-dsDNA has proven the most successful approach in achieving significant linkages in genome-wide scans. This suggests lupus might comprise a set of different diseases with varying underlying genetic determinants. In clinical practice, laboratory investigation of patients with possible lupus proceeds from high-sensitivity, low-specificity tests to more specific investigations. Similarly, once an ANA-positive strain is identified in the primary screen and heritability established, in-depth phenotyping of ANA-positive strains is necessary to establish a precise clinical picture of lupus abnormalities caused by each mutation, enabling direct comparisons with the laboratory results from different lupus patients and informing the human clinical analyses about additional tests that may help resolve genetic subsets of lupus (Figure 3).

For detailed clinical pathology, blood is taken from each strain of mice between 10 and 18 weeks of age, and the following tests are performed. (1) Full blood count: Blood is examined on a hematology analyzer (five parameter white cell differential) calibrated for analysis of mouse blood. Autoimmune anemia, thrombocytopenia and leukopenia all occur in lupus as a consequence of autoantibody production. In strains where cytopenia is identified, serum transfers are performed to determine whether these are antibody mediated. As an example, *san roque* mice have been shown to exhibit autoimmune thrombocytopenia since it can be reproduced in wild-type (WT) littermates upon transfer of serum from affected mice. (2) Extended autoantibody analysis: Additional tests are also performed for antibodies that are characteristic of lupus. Serum is screened on *Crithidia luciliae* substrate for high-affinity IgG dsDNA antibodies. Tests for IgG antibodies to extractable nuclear antigens (SS-A, 52 and 60 kDa antigens, SS-B,

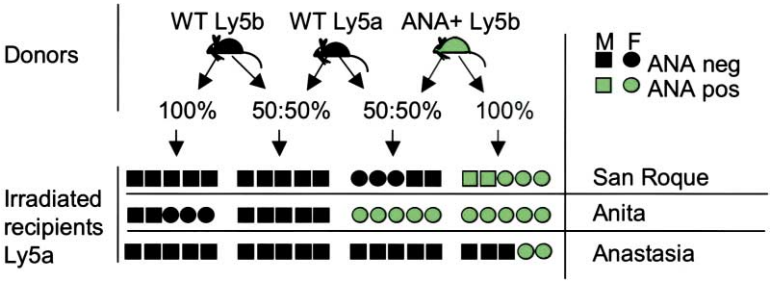

Figure 5. Bone Marrow Chimeras Identify Different Routes to ANA Development

Examples of mixed bone marrow chimeras constructed using WT donor bone marrow and/or bone marrow from ANA-producing ENU strains. All recipients of *Anita* bone marrow develop autoantibodies whereas WT bone marrow corrects the autoantibody production in mixed WT/*san roque* bone marrow chimeras. Strikingly, only female recipients of *Anastasia* bone marrow develop autoantibodies.

Sm, RMP, Ribosomal P, and Jo-1) are performed by immunoblot. Already, ANA-positive strains have been shown to produce antibodies to dsDNA, SS-A, and Ribosomal P antigen. (2) Renal pathology: Evidence is sought using several approaches. Serum is analyzed for hypoalbuminemia and hyperlipidemia (signs of nephrotic syndrome). This is combined with testing for proteinuria by urinalysis. Significant proteinuria is further characterized by urine electrophoresis. Finally, kidneys are examined for changes of lupus nephritis by light microscopy (hematoxylin and eosin, silver staining) and electron microscopy, and immunofluorescence for immunoglobulin deposition. Three of the strains tested so far have shown renal damage with reabsorption droplets in tubules, amyloid-like deposits in the glomeruli, and focal thickening of glomerular basement membranes due to immune-complex deposition and microthrombi in the capillaries. (4) Skin pathology: Lupus is characterized by immunoglobulin deposition at the dermoepidermal junction and a lymphocytic infiltrate. Skin is taken at necropsy and examined by histology for lupus-like infiltrate and by immunofluorescence for lupus band.

The approach used to delineate what cellular and molecular pathways are altered by each new ANA variant is summarized in Figure 3. The first step in separating out primary from secondary effects of the variant gene is to identify whether the defect in each strain resides in the hematopoietic system and, if so, whether it is T and/or B cell intrinsic. This is determined by constructing radiation chimeras in which RAG-deficient recipients are reconstituted with bone marrow from mutant or WT congenic mice, or with a 50:50 mixture of mutant and WT marrow. The congenic WT donor cells are allotype marked with $Ly5^a$ or with $Thy1^a$ and IgH^a to enable dissection of panhematopoietic, T and B cell defects, respectively. Twelve weeks after reconstitution, mice are bled and analyzed by flow cytometry and serum antibody testing. FACS experiments are used to determine whether T or B cell activation is confined to the mutant-derived cells, indicating a cell-intrinsic defect.

Figure 5 illustrates the functional distinction between three ANA strains revealed by the mixed bone marrow chimera approach. In recipients of bone marrow from the *Anita* line, ANAs develop when all or only 50% of the hematopoietic cells carry the variant gene, indicating that the defect acts cell autonomously in blood cells. Mutations in T and B cell inhibitory molecules such as Fas or SHP-1 behave in this way. By contrast, in recipients of marrow from *san roque*, mixed wild-type/mutant bone marrow chimeras do not develop ANAs whereas chimeras with 100% mutant blood cells are ANA positive. This kind of correction by wild-type blood cells is typically observed for defects in *trans*-acting factors such as Fas-ligand or regulatory T cells. A third pattern is illustrated by *Anastasia*, a line that exhibits a strong female bias in the susceptibility to autoantibodies. Only the female recipients of *anastasia* bone marrow develop ANAs, indicating an interaction between a blood cell acting gene variant and the recipient's sex. Strong female to male bias (9:1) in ANA production typically occurs in human systemic autoimmune disease and is thought to be a consequence of the effects of sex hormones on immune regulation, but the absence of adequate animal models of lupus showing the typical female bias has been a significant obstacle in elucidating the mechanism (McMurray, 2003).

In order to test whether there are T cell- or B cell-intrinsic proliferation or activation defects, CFSE-labeled mutant and Ly5 congenic wild-type T cells and B cells are cocultured in the presence of various stimuli that act through antigen receptors, Toll-like receptors, and costimulatory molecules. Expression of activation markers and proliferation kinetics are assessed by flow cytometry. This assay provides information about the requirement for costimulation in mutant cells, a key question since known defects that lead to T cell hyperactivation and ANA development abrogate this requirement (Bachmaier et al., 2000; Chiang et al., 2000). If intrinsic T and B cell defects are excluded, the next step is to look for potential APC-intrinsic defects. These are initially also studied with mixed bone marrow chimeras by looking at macrophage and dendritic cell activation markers. Finally, if B cell, T cell, and APC-intrinsic defects are excluded, serum from affected mice is analyzed for complement or serum amyloid P (SAP) deficiencies that can impair the clearance of DNA or apoptotic cells. In this instance, autoimmune susceptibility may reside in nonhematopoietic tissues such as the liver, and this is tested by reconstituting lethally irradiated mutant recipients with wild-type Ly5a congenic bone marrow.

A Web-Based Public Repository for Data on Immune Variant Strains

The data from all ENU-induced ANA-producing mouse strains identified so far through a Wellcome Trust funded program, resulting from the implementation of the immunization and systemic autoimmune screens described above is accessible at the following website: www.apf.edu.au/resources/wt/. The aim is to make the information obtained from these strains freely available to the scientific community and facilitate the transfer of estab-

lished mutant mouse strains to interested groups. The website will provide information on heritability, phenotypic characterization, and stage of gene identification (chromosomal linkage, fine mapping, sequencing of transcripts in interval) for each strain. All recipients of the strains are expected to deposit in the database any new data on the strains, thus building up an openly accessible base of knowledge about immune regulatory genes and processes. Further development of immunological screens and screening of new libraries to generate other variant gene resources is underway in specific projects supported by the Juvenile Diabetes Research Foundation and the Australian NHMRC, and in an ANU-UCSF collaboration supported by the National Institutes of Health.

Mapping and Sequencing of Mutations

Once over the hurdle of identifying a new immune variant and establishing a true-breeding line, the strategy for mutation identification involves a well-established generic process of linkage mapping through intercrosses with another inbred mouse strain (Jun et al., 2003; Nelms and Goodnow, 2001). Choosing the right outcross strain is critical for the identification of mutations that drive autoimmune phenotypes. Initial efforts at mapping lupus-prone strains by outcrossing to the NOD background proved largely unsuccessful since the NOD strain has an inherent higher susceptibility to develop antinuclear antibodies and a large number of phenocopies was found when looking for other traits such as lymphadenopathy and hypergammaglobulinemia. Initial observations from mapping strategies using CBA/H as the mapping partner for ANA strains show some of the autoimmune phenotypes display decreased clinical and serological severity, which is useful when it prolongs longevity to the age of screening but can also be problematic if it suppresses detection of the trait altogether. In the latter case, another inbred strain such as Balb/c or DBA/2 can be used.

Any evidence for dominant inheritance of ANA traits is tested in F1 mice, and in this case the mapping cross proceeds as an N2 outcross of the F1s to CBA. Recessive mutants are mapped by intercrossing F1 siblings to yield F2 intercross offspring. Initial linkage to a 10–20 cM (20–60 Mb) chromosomal segment is established by testing the first 200 N2 or F2 mice for each strain at 10–18 weeks of age for the presence of autoantibodies and any other specific immune or clinical characteristics identified in that particular strain. Control crosses are needed to ensure that suitable cutoffs are used to distinguish mutants from other sources of genetic variability in the cross.

Two alternative strategies have proved useful for initial chromosomal linkage assignment of immune variants. The least labor-intensive method involves pooling equal amounts of DNA from 15 to 25 mice displaying ANA's or other immune defects. The one pooled sample is then tested for a high proportion of B6-derived chromosomal regions by PCR amplification and agarose gel electrophoresis of SSLP markers spaced at $\sim$20 cM (i.e., 3–4 markers per chromosome). Alternatively, if insufficient affected animals are obtained in the initial cross, linkage can be determined by typing as few as six affected animals individually with SSLP markers at the ends of each chromosome, using the method of Beier (Neuhaus and Beier, 1998). The linked chromosomal interval is then confirmed and narrowed by individually typing each of the initial affected animals with additional SSLP markers spanning the region of linkage. Once a linked marker has been identified, this greatly facilitates distribution of strains, since recipient laboratories can use simple PCR tests instead of specialized serological progeny testing to identify carriers and homozygotes during colony establishment and experimental analysis.

To reduce the linkage region to 1–2 Mb, subsequent N2 or F2 mice are typed at weaning for two flanking markers, and useful recombinants within the interval are kept to localize the point of crossover with additional SSLPs or SNPs within the interval, for phenotypic analysis once they are 10–18 weeks old and for progeny testing as needed. During this phase, when there may be $>$100 genes within the linkage interval, mRNAs for any strong candidate genes are fully resequenced. Once the region has been reduced to $\sim$1 Mb or a region containing less than 30 genes, all the mRNAs encoded in the interval are sequenced. By PCR amplification of cDNA in 700 bp overlapping fragments, gel purification, and sequencing, we identify which of these transcripts is altered in coding (75% of ENU mutants) or mRNA splicing sequences (25% of ENU mutants). In over 40 mutant strains arising from the ANU mutagenesis effort, all the mutations have been assigned to one or other of these categories.

Candidate-Based Identification of Human Lupus Alleles

Recent evidence from genome scans has suggested that the success of identifying disease links to causative gene loci can be enhanced once lupus patients are stratified according to their clinical manifestations (e.g., renal, neuropsychiatric, hematological manifestations) (Martin and Reichlin, 1996; Scofield et al., 2003). If this strategy is combined with a powerful and unbiased program of candidate gene discovery, where the genotype-phenotype association is defined in detail in murine models, then novel disease pathways are likely to emerge. Once the mutated gene is identified in each autoimmune-prone strain, the orthologous human gene is automatically identified by reference to the Ensembl human and mouse genome sequence database. Related genes and protein domains are detected from the database and by well-established p-blast, psi-blast, and threading algorithms available on the net. This information may point to the cellular subsets and signaling pathways that may be dysregulated by each mutation and highlight potential connections between the human genes identified and susceptibility to autoimmune disease that may have been found in linkage studies. Importantly, the Ensembl annotated human genome sequence enables immediate design of primers to amplify the exons of the human candidate lupus genes and sequence these for variants that may be present in subsets of lupus patients.

Too little is known about the pathogenesis of lupus to predict whether it will represent a collection of disorders arising from a range of rare gene variants with strong

individual effects (as appears to be the case with NOD2 truncations in Crohn's disease) or from complex additive combinations of common gene variants with weak individual effects. The recent identification of a rare gain-of-function variant in CD40 in a subset of lupus patients (Grammer et al., 2004) is consistent with the former. Even if strong variants in the human counterparts of Mendelian autoimmune-susceptibility mouse alleles only account for a minority of lupus cases, they can still have a broad impact. First, these discoveries refine the search for additional candidate genes encoding other proteins in the same pathway, such as the other components of the VDJ recombination or IL2Rγ pathways in inherited immunodeficiency (Corneo et al., 2001; Notarangelo et al., 2000), or of the Fas pathway in ALPS (Fisher et al., 1995; Wang et al., 1999). Second, identification of relatively rare disease-associated alleles can have significant diagnostic value (e.g., BRCA1 in breast cancer [Huttley et al., 2000]). Third, once the pathogenesis of severe phenotypes is elucidated in relatively rare patients, rational approaches to therapy with much broader application may become apparent. This is best illustrated by the development of statin lipid-lowering therapy for common forms of atherosclerosis, which was based upon the recognition of defects in cholesterol metabolism as the cause of premature atherosclerosis in rare, early onset patients with homozygous LDL receptor defects (Goldstein and Brown, 1990). The uniquely high signal-noise ratio for discovering variants in autoimmune regulatory genes by controlled variation of the mouse genome sequence with ENU, coupled with the syntenic sequences of humans and mice and the tools for analyzing immune regulatory processes in mice, appear to make this a particularly rich strategy for developing specific treatments for lupus and other immunological diseases.

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